Appeal Brief under 37 C.F.R. §41.37		
	Attorney Docket No.	**3032-101**
	First Named Inventor	Igor Stagljar
	Title: **Method and kit for detecting membrane protein-protein interactions**	
	Application Number	10/509,507
	371(c) Date	December 22, 2004
	Group Art Unit	1636
	Examiner Name	Michele K. Joike

MAIL STOP APPEAL BRIEF PATENTS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This is an appeal to the Board of Patent Appeals and Interferences from a final Office Action mailed on October 3, 2007 in which the Examiner rejecting claims 48 to 65, 68, 70 to 72 and 75 to 76 as modified per advisory action of January 24, 2008 entering amendments overcoming the rejections of claims 75 to 76.

Applicants timely filed a Notice of Appeal in this case on March 24, 2008. Accordingly, a brief in support of the appeal is due by Tuesday, May 27, 2008, the first business day after Monday, May 26, 2008 (Memorial Day) and this Brief is therefore deemed to be timely filed (37 C.F.R. §41.37(a)(1)). The fee due in connection with the filing of this Brief is submitted herewith (37 C.F.R. §41.20(b)(2)).

No additional fees are believed to be due in connection with the filing of this Brief. However, the Commissioner is authorized to charge any fees that might be necessary for consideration of this filing, or credit any overpayments, to Deposit Account No. 50-3135. Any extensions of time that might be required are respectfully requested herewith.

1. Real Party in Interest

The real party in interest in this appeal is Dualsystems Biotech AG, the assignee of record.

2. Related Appeals and Interferences

No related appeals or interferences are known to Appellants or Appellants' legal representative which will directly affect or be directly affected by or have bearing on the Board's decision in this appeal.

3. Status of Claims

Claims 48 to 76 are pending in the application. Claim 69 is withdrawn from consideration. Claims 66 and 67 and 73 to 74 are objected to for being dependent on rejected base claims, but have been stated to be allowable if rewritten in independent form including all limitations of the base claim and any intervening claims. Claim 75 and 76 are also objected to and have no rejections outstanding against them. Claims 48 to 65, 68 and 70 to 72 stand twice rejected. Since, as of the date of this appeal, none of the amendments to claims 66, 67, 73, 74, 75 and 76 have been entered, all of the rejected and objected to claims, namely claims 48 to 68 and 70 to 76 are on appeal.

4. Status of Amendments

The amendments to claim 75 filed on December 3, 2007, which overcame the 35 USC §112, second paragraph rejection of claims 75 and 76, were entered for purposes of appeal per Advisory Action of January 24, 2008 and now stand objected to. A further amendment was filed with the Notice of Appeal on March 24, 2008 to rewrite claims 66 and 67 and 73 to 74 in independent form including all limitations of the base claim and any intervening claims. The amendment was denied entry. After a telephone interview with Examiner Joike on April 24, 2008 to discuss amendments acceptable to the Office, a further amendment was filed on that April 24, 2008 to amend claim 66 and 67 and 73 to 74 accordingly. Per PAIR, as of the date of filing this appeal brief, no further advisory action, entering or denying entry of these amendments has issued. An amendment in accordance with 37 CFR §41.33 (b)(2) is filed with this appeal to further bring claim 75 into independent form and to thus overcome the objections against claims 75 and 76.

5. Summary of Claimed Subject Matter

The claimed invention is a method for detecting interactions of a first membrane protein or part thereof expressed by a first vector (bait vector) with a second protein or parts thereof expressed by a second vector (prey vector). The detection is based on the reconstitution of a protein that is involved intracellular protein degradation in a host cell comprising the bait and prey vector. This reconstitution is detectable. An example of a

protein that is involved in intracellular protein degradation is ubiquitin. An illustration of the split-ubiquitin technique, which is based on the ability of NbM and CbM, the N- and C-terminal halves of ubiqitin, to assemble into quasi-native ubiquitin is shown in Fig. 1 of the present disclosure.

The claimed method provides a bait vector. This bait vector comprises a gene encoding the first membrane protein or part thereof which is attached to the DNA sequence of a first module encoding a first protein sequence involved in intracellular protein degradation. Such a first protein sequence is, e.g., a C- terminal part of split ubiquitin. The bait vector also includes a DNA-sequence for a transcriptional activator (The terms "bait" and "bait vector" are defined on page 7, first and second paragraph of the disclosure, preferred features of certain bait vectors and specific examples of bait vectors are provided under the heading "Bait vector" starting on page 22 and ending on page 37 of the specification).

The claimed method also provides for a prey vector. The prey vector comprises a gene encoding the second protein or part thereof which is attached to the DNA sequence of a module encoding a second protein sequence involved in intracellular protein degradation. Such a second protein sequence is, e.g., N-terminal part of split ubiquitin. (The terms "prey" and "prey vector" are defined on page 7, third and fourth paragraph of the disclosure, preferred features of certain prey vectors and specific examples of prey vectors are provided under the heading "NbM library vectors (prey vectors)" starting on page 38 and ending on page 41 of the specification).

The bait vector and the prey vector are introduced into a host cell which contains at least one detectable gene which can be activated via the transcriptional activator of the bait vector. An interaction between the expressed first (membrane bound) and second protein that the method seeks to detect leads to an interaction of the first protein sequence encoded by the first module (e.g., an C-terminal part of split ubiquitin) and the second protein sequence encoded by the second module (e.g., an N-terminal part of split ubiquitin). The later interaction results in the reconstitution of a protein involved in protein degradation, e.g., split ubiquitin, which in turn leads to the proteolytic separation of the transcriptional activator. The so separated transcriptional activator activates the detectable gene of the host cell. Activation of this detectable gene allows detection of the interaction between the first membrane protein of interest and the second protein. Importantly, both the bait vector and the prey vector are maintained episomally.

The invention is also directed at a kit for detecting binding between a first membrane bound protein or part thereof and a second protein or part thereof. The kit comprises an episomally maintained first vector (bait vector), for the membrane protein to be tested as

well as the transcriptional activator, an episomally maintained second vector (prey vector) and a host cell. The host cell corresponds to the one described above. The first vector (bait vector) comprises a first site for a nucleic acid encoding a first membrane protein or part thereof and a DNA sequence for a first module encoding a first protein sequence involved in intracellular protein degradation. Such a first protein sequence is, e.g., a C-terminal part of split ubiquitin. The first vector (bait vector) also comprises a nucleic acid for a transcriptional activator. The second vector (prey vector) comprises a second site for a nucleic acid encoding a second protein or part thereof and a DNA sequence for a second module encoding a second protein sequence involved in intracellular protein degradation. Such a second protein sequence is, e.g., N-terminal part of split ubiquitin. The kit detects the binding between the first membrane bound protein and the second protein.

6. Ground of Rejection to be reviewed on Appeal

The grounds of rejection to be reviewed on appeal are as follows.

Whether claims 48 to 65 and 71 and 72 are anticipated under 35 USC §102(b) by Stagljar et al. (PNAS 95:5187-92, 1998, in particular p. 5187, 5191 and Fig. 2; hereinafter "Stagljar").

Whether claims 68 and 70 are unpatentable under 35 USC §103(a) over Stagljar in view of Ehrhard et al. (Nature Biotech. 18: 1075-1079, 2000; hereinafter "Ehrhard").

7. Argument

A. STAGLJAR DOES NOT DISCLOSE ALL ELEMENTS RECITED IN CLAIMS 48 to 65, 71 AND 72 AND THUS DOES NOT ANTICIPATE CLAIMS 48 to 65, 71 AND 72 UNDER 35 USC §102(b).

Claims 48 to 56:

Claim 48 is an independent method claim. Claims 49 to 56 are dependent on claim 48.
The Office alleged that Stagljar discloses all elements of the detection method of claim 48. In particular, the Office alleged that Stagljar teaches that the bait and the prey vector are maintained episomally. The Office referred in particular to pages 5187, 5191 and Fig. 2 of Stagljar. In the Advisory Action of January 24, 2008 the Office expanded on its reasoning underlying the statement that Stagljar teaches maintaining a bait vector episomally. In particular the Office further explained, making reference to page 5190, second paragraph, that Stagljar teaches that NubG-Alg5p can be expressed from a high or a low copy plasmid.

Claim 48 requires:

"(b) providing, as part of a bait vector, . . . coding . . . for a first membrane protein or part

thereof . . . a first protein sequence involved in intracellular protein degradation and a transcriptional activator,

. . .

(d) introducing the bait vector and the prey vector into the host cell . . ., wherein both the bait vector and the prey vector are maintained episomally;

. . .

(f) detecting said interaction between said first membrane bound protein or part thereof and said second protein or part thereof." *(emphasis added)*

In the analysis below, appellants will show that, while Stagljar teaches that a plasmid encoding an N-terminal part of split ubiquitin (e.g., "NubI") can be maintained episomally, Stagljar does NOT teach that the plasmid encoding the membrane protein of interest, as well as "Cub" and, in particular also, a transcriptional activator and thus the plasmid disclosed in Stagljar containing elements of the claimed "bait vector" of the present invention, namely pRS305 (Δwbp1-Cub-PLV), is maintained episomally. At the same time it will become clear that plasmids encoding an N-terminal part of split ubiquitin, e.g., NubG-Alg5p as specifically mentioned by the Office, do not contain the elements of a bait vector, but rather resemble the "prey vectors" of the present invention. Accordingly, appellants will show that Stagljar does not disclose the highlighted element of claim 48 under (b) and (d) outlined above. In fact, appellants will show that only upon integration of pRS305 (Δwbp1-Cub-PLV) into the yeast genome, the full fusion protein whose interaction Stagljar tests, will be expressed (see claim 48 under (f)). While not directly pertinent to an anticipation rejection, appellants will also point out, that Stagljar provides at least one indication that suggests that an episomal maintenance of this vector (pRS305 (Δwbp1-Cub-PLV)) is considered undesirable.

Appelants' argument is supported by e.*g.*, page 5187, right column ("col."), last paragraph, under "Strains, Media" *("...pRS305 (Δwbp1-Cub-PLV) was integrated at the single SpeI site into the WBP1 gene...")* and page 5189, left col., l. 7 to 10, *("The WBP1-Cub-PLV fusion gene was generated by site-directed integration of a PLV cassette containing the 5'- truncated Δwbp1 gene (Δwbp1-Cub-PLV) into the genomic WBP1 locus")* and the fact that, as others vectors in this series, pRS305, whose structure is detailed on page 5188, left col., starting on line 14, is an integrative vector, which does not contain a yeast episomal origin of replication. Furthermore, only upon site specific integration of pRS305 (Δwbp1-Cub-PLV) into the WBP1 locus of the yeast genome, the yeast genome will provide, next to the Wbp1 promoter, the N-terminal part of the Wbp1 to create a full Wbp1-Cub-PLV fusion protein, whose interaction is then tested (see p. 5188, left col., l. 15 (" *the . . . insert within pRS305 (17) encodes amino acids 251-430 of Wbp1p (Δwbp1)*"; p. 5189, left col., l. 7 to 10 "*The WBP1-Cub-PLV fusion gene was generated by site-directed integration of a PLV cassette containing the 5'- truncated Δwbp1 gene (Δwbp1-Cub-PLV) into the genomic WBP1 locus*"; and p.

5189, right col., starting on line 6 entitled "*Interaction of Wbp1-Cub-PLV with Ost-Nub and Nub-Alg5p*").

The specification defines the term "bait" on page 7, first paragraph, as a fusion of a polypeptide and one or more other polypeptides, one of which is a first protein sequence involved in intracellular protein degradation such as CbM. As the name suggests, the bait can be used to investigate interactions between the bait and one or several preys.
CbM, as noted, for example, on page 15 of the specification is the C-terminal half of Ub (Ubiquitin) which assembles with NbM, the N- terminal half of Ub into quasi-native Ubiquitin.
The specification defines the term "BAIT VECTOR" as a nucleic acid construct which contains sequences encoding "the bait" and regulatory sequences that are necessary for the transcription and translation of the encoded sequences by the host cell (page 7, second full paragraph). The "bait vector" is said to preferably also encode the activator of the host reporter gene(s). The claimed invention requires a "transcriptional activator" as part of the bait vector.

The specification defines the term "prey" as a fusion between a polypeptide and one or more other polypeptides, one of which is a second protein sequence involved in intracellular protein degradation such as NbM (page 7, third paragraph).
NbM, as noted, for example, on page 15 of the specification is the N-terminal half of Ub (Ubiquitin) which assembles with CbM, the C- terminal half of Ub into quasi-native Ubiquitin.

The specification defines the term "PREY VECTOR" as a nucleic acid construct which contains sequences encoding the "prey" and regulatory sequences that are necessary for the transcription and translation of the encoded sequences encoding by the host cell (page 7, fourth paragraph).

The specification also defines, on page 10, third full paragraph, "first protein sequence involved in intracellular protein degradation" and "second protein sequence involved in intracellular protein degradation" as used in the claims. These terms are said to mean parts of a protein which, when brought together in a host cell, e.g. by the interaction of the first protein to be tested and the second protein to be tested, reconstitutes a structure which is capable of activating an intracellular protein degradation machinery such as the ubiquitin depending proteases. Thus, for a reconstitution to take place, both of these protein sequences, e.g., NbM and CbM have to be present.

THE TEACHINGS OF THE STAGLJAR REFERENCE

Stagljar describes his work with a system employing NubI, amino acids 1-34 of ubiquitin and related N-terminal portions of ubiquitin, such as NubA and NubG and Cub, amino acids 35 to 76 of ubiquitin in his system (p. 5187, left col., l. 16 -21 of the paragraph following the abstract). Reconstitution of the those Nubs and Cub are required for Stagljar's system to work (see, e.g., Fig. 2 (B) and (D)). Stagljar's vector

pRS305 (Δwbp1-Cub-PLV) contains sequences for Cub and the transcription factor Protein A-LexA-VP16 ("PLV"), which was used as a "reporter molecule" (see Stagljar, page 5187, right col., l. 22 to 27 of the first full paragraph). Thus, pRS305 (Δwbp1-Cub-PLV) contains elements required by the "bait vector" or "first vector" of the presently claimed invention. Stagljar's vectors pRS314 and pRS304 contain Nub or permutations thereof. Stagljar tested the full protein Wbp1, a type I transmembrane protein, for its interaction with other proteins (see page 5188, right col., lines 3 to 4 of the last paragraph (entitled "Results") which is, in part, provided by the Cub-PLV (PLV = transcription factor) encoding vector. Alg5p, which is part of one of the Nub (NubI, NubA, NubG) containing vectors pRS314 (NubI-ALG5, NubA-ALG5, NubG-ALG5), is a type II transmembrane protein (see page 5187, right col., lines 10 to 17 of the first full paragraph), while Ost1p, which is part of Nub (NubI, NubA or NubG) containing vectors pRS306, (Δost1-NubI, Δost1-NubA, Δost1-NubG) is, like Wbp1p a type I transmembrane protein (see page 5187, right col., first full paragraph, l. 13 to 14).

Some of the Nub encoding plasmids of Stagljar are constructed as 2-µm plasmids, e.g., pNubI-ALG5, pNubA-ALG5, pNubG-ALG5 (see page 5188, left col., last full paragraph), whereas others are integrative plasmids, e.g., Δost1-NubI, Δost1-NubA, Δost1-NubG (see page 5188, col. bridging paragraph). Thus, Stagljar discloses both, Nub containing vectors that are integrated (see, page 5189, col.1, l. 16 018) and, Nub containing plasmids which are, indeed maintained episomally (see, e.g., also page 5189, left col., last five lines). The latter, namely Nub containing plasmids which were maintained episomally, lead to overexpression of the respective Nub-fusion protein, which is, in the context of the Nub containing vectors in question, often desirable (e.g., Stagljar, page 5189, right col., first 5 lines and page 5190, left col., last three lines to right col., line 3).

However, Stagljar does not disclose anywhere that both the Nub and the Cub-PLV (PLV = transcription factor) plasmids are maintained episomally. Moreover, the Cub containing plasmid pRS305 (Δwbp1-Cub-PLV) does not contain the entire the entire WBP1 open reading frame. Rather the plasmid contains a 5'-truncated "Δwbp1 gene" (see page 5188, left col., l. 15) and, to put it in Stagljar's words, the WBP1-Cub-PLV fusion gene is "generated by site-directed integration of a 5'-truncated Δwbp1 gene (Δwbp1-Cub-PLV) into the genomic WBP1 locus." (see page 5189, left col., l. 7 to l. 10). Thus, the yeast genome provides, upon integration, next to the Wbp1 promoter to drive expression (see description of pRS305 on page 5188, left col. starting on page 14), the N-terminal part of the Wbp1 ORF to provide a full Wbp1-Cub-PLV fusion protein. Stagljar then tests this full Wbp1-Cub-PLV fusion protein for interaction with Ost1-Nub and Nub-Alg5p . (see page 5189, right col., starting on l. 6).

Stagljar also indicates what might amount to a rationale for the absence of an episomally maintained bait vector: In particular, on page 5191, right col., l. 9 to 10, Stagljar states that "overexpression of WBP1-Cub-PLV . . . results in gene activation in the absence of Nub." Such so called "self activation" is highly undesirable, since a protein interaction cannot be detected anymore as required in the element (f) of claim 48 (see emphasis above).

Accordingly, appellants submit that Stagljar does not disclose that the bait and prey vector are maintained episomally. Rather, while one of the plasmids of Stagljar, namely the plasmid that can be characterized as the "prey vector" (Nub expressing plasmid) can be maintained episomally, the other plasmid, which is a prerequisite for the interaction tested by Stagljar and which contains elements of the "bait vector" of the present invention is integrated to allow expression of its "bait." This plasmid does not contain regulatory sequences that are necessary for the transcription of the encoded sequences by the host cell in accordance with the definition of a bait vector of the present invention. Accordingly, appellants submit that Stagljar does not disclose all elements of the claimed invention as required for an anticipation rejection (MPEP §2131).

Claims 66 and 67:

Claims 66 and 67 are directly or indirectly dependent on claim 48 and have been objected to, but have been found allowable if rewritten in independent form including all the limitations of the base claim and any intervening claims. Accordingly, no arguments are presented with respect to these claims.

Claims 57 to 62, 64, 65 (Kit Claims):

Claim 57 is an independent kit claim. Claims 62, 64 and 65 are dependent on claim 57.
The Office alleged that Stagljar discloses all elements of the kit of claim 57.
In particular, the Office rejected these claims for the same reasons it rejected the method claims and which have been discussed above.
Claim 57 requires:

> "(b) a first vector (bait), which is maintained episomally, . . . receive a first nucleic acid . . . becomes attached to the DNA sequence of a first module encoding *inter alia* a first protein sequence involved in intracellular protein degradation, . . . further comprising a nucleic acid for a transcriptional activator and a promoter;
> (c) a second vector (prey), which is maintained episomally" *(emphasis added).*

As outlined above, Stagljar does not disclose that both the first (bait) and second (prey) vectors are maintained episomally. Rather, the plasmid expressing Cub and comprising the nucleic acid for the transcriptional activator, namely pRS 305 (Δwbp1-Cub-PLV), is integrated. As discussed above, pRS305 (Δwbp1-Cub-PLV) contains a 5'-truncated "Δwbp1 gene" and, to put it in Stagljar's words, the "WBP1-Cub-PLV fusion gene was generated by site-directed integration of A 5'-truncated Δwbp1 gene (Δwbp1-Cub-PLV) into the genomic WBP1 locus." (see page 5189, left col., l. 7 to l. 10). Considering the structure of pRS305, which is detailed on page 5188, left col. starting on line 14, next to the N-terminal part of WBP1, the promoter that drives the expression, is provided by the yeast genome. The plasmid expressing Nub

does not contain a promoter. As also discussed above, Stagljar discloses a system containing a specific bait membrane protein (Wbp1p) and specific prey membrane proteins (Alg5p, Ost1p). Stagljar does not disclose vectors having sites for receiving nucleic acids coding for such proteins or parts thereof.
Accordingly, appellants submit that Stagljar does not disclose that both the first vector (bait) and the second vector (prey) are maintained episomally. Appellants also submit that Stagljar does not disclose a first vector comprising a promoter. Furthermore, appellants also submit that Stagljar does not disclose a first and second vector that is adaptable for receiving proteins of interest.

Accordingly, appellants submit that Stagljar does not disclose all elements of the claimed invention as required for an anticipation rejection (MPEP §2131).

Claim 63:
Claim 63 is dependent from claim 57 and requires that the promoter of the first vector (bait) in the kit is a CYC1 promoter or a CUP1 promoter. Claim 63 was rejected by the Office for the same reasons as claim 48.

While the CUP1 promoter is mentioned to be part of the vector containing NubI (see page 5188, left col., para., 3, lines 1 to 2, it is not mentioned in the context of the plasmid having features of the first vector (bait), that is pRS305, which contains Cub and the nucleic acid for a transcriptional activator. In fact, pRS305, which is described on page 5188, left col., starting on line 14, does not comprise any promoter sequences. As discussed above, an appropriate promoter sequence is provided by the yeast genome together with the N-terminal part of the WBP1 gene upon integration into the yeast genome.
Accordingly, Stagljar does not disclose this feature of claim 63 providing in the context of this claim a further reason why Stagljar does not meet the requirements of an anticipating reference (MPEP §2131).

Claims 71 and 72:
Claim 71 is dependent from claim 48 and requires that the bait vector is a low copy vector.
Claim 72 is dependent from claim 71 and requires that the bait vector is present at one to two copies per cell.

The Office expressed the opinion that Stagljar teaches CEN/ARS which is a low copy vector present in only 1 to 2 copies per cell.

While this is correct (see e.g., Fig 3 of Stagljar) expression from a low copy vector is only disclosed in the context of vectors expressing Nub, and not in the context of the plasmid

encoding Cub and a transcriptional activator and thus the plasmids comprising elements of a bait vector, namely pRS305 (Δwbp1-Cub-PLV).

Thus, since claims 71 and 72 clearly refer to a vector for the membrane protein or part thereof to be tested, and the transcription activator ("bait vector"), Stagljar also does not disclose the further elements required by claims 71 and 72 providing a further reason why Stagljar does not meet the requirements of an anticipating reference of claims 71 and 72 (MPEP §2131).

Claims 73 and 74:
Claims 73 and 74 are directly or indirectly dependent on claim 57 and have been objected to, but have been found allowable if rewritten in independent form including all the limitations of the base claim and any intervening claims. Accordingly, no arguments are presented with respect to these claims.

Claims 75 and 76:
Claims 75 and 76 are directly or indirectly dependent on claim 57 and have been objected to. While not explicitly found allowable if rewritten in independent form including all the limitations of the base claim and any intervening claims, no other base for objection to or rejection of claims 75 and 76 is on file. Accordingly, no arguments are presented with respect to these claims.

B. THE OFFICE HAS NOT SET FORTH A PRIMA FACIE CASE HOW THE STAGLJAR AND EHRHARD REFERENCES RENDER CLAIMS 68 AND 70 UNPATENTABLE UNDER 35 USC §103(a).

Claims 68 and 70:
Claim 68 is dependent on claim 57 and is directed at a method for screening compounds for their ability to interfere with protein-protein interactions using the kit of claim 57.
Claim 70 is dependent on claim 68 and further specifies that the compound is a pharmaceutical drug.

The Office alleged that Stagljar teaches all limitations of kit claim 57, but acknowledged that Stagljar does not teach a method for identifying pharmaceutical drugs for their ability to interfere with protein-protein interactions. However, the Office expressed the opinion that Ehrhard teaches a method of identifying compounds for their ability to interfere with protein-protein interaction.

Stagljar and its deficiencies with respect to claim 57 have been discussed above.

Ehrhard's method relies on G-protein fusions to monitor integral membrane protein-protein interactions. The Office has not provided any reasoning how Ehrhard addresses the deficiencies of Stagljar detailed above. Accordingly, no *prima facie* case of obviousness has been established by the Office (MPEP §2142, MPEP §2143). Appellants submit that Ehrhard does not make obvious these deficiencies of Stagljar.

Conclusion

Having set forth the factual and legal basis which supports the patentablity of the claims on appeal, it is respectfully submitted that claims 48 to 68 and 70 to 76 are allowable. Accordingly, it is respectfully urged that the Board reverse the Examiner's rejection thereof.

Respectfully submitted,

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8. Claim Appendix

48. A method for detecting an interaction between a first membrane bound protein or part thereof and a second protein or part thereof, which is either membrane bound or soluble, the method comprising:
(a) providing a host cell containing at least one detectable gene (reporter gene) having a binding site for a transcriptional activator, such that the detectable gene expresses a detectable product when the detectable gene is transcriptionally activated;
(b) providing, as part of a bait vector, a first chimeric gene under the control of a promoter, said first chimeric gene being expressed in said host cell and coding *inter alia* for a first membrane protein or part thereof which gene is attached to the DNA-sequence of a first module encoding *inter alia* a first protein sequence involved in intracellular protein degradation and a transcriptional activator, said first protein or part thereof to be tested whether it can interact with a second protein or part thereof;
(c) providing, as a part of a prey vector, a second chimeric gene under the control of a promoter that can be expressed in said host cell, the second chimeric gene coding *inter alia* for a second protein or part thereof which is either membrane bound or soluble and which gene is attached to the DNA sequence of a second module encoding *inter alia* a second protein sequence involved in intracellular protein degradation;
(d) introducing the bait vector and the prey vector into the host cell such that an interaction between the expressed first and second proteins and/or their parts can take place, which interaction leads to an interaction of the first protein sequence of the first module and the second protein sequence of the second module which interaction in turn leads to activation of an intracellular protease and proteolytic separation of the transcriptional activator, wherein both the bait vector and the prey vector are maintained episomally;
(e) determining whether the detectable gene of the host cell has been activated by the transcriptional activator; and
(f) detecting said interaction between said first membrane bound protein or part thereof and said second protein or part thereof.

49. The method according to claim 48, wherein the host cell is a yeast, a bacterium or a mammalian cell.

50. The method according to claim 49, wherein the yeast is *Saccharomyces pombe* or *Saccharomyces cerevisiae*.

51. The method according to claim 48, wherein the detectable gene is activated by an activator comprising a short tagging module.

52. The method according to claim 48, wherein the detectable gene is activated by the artificial transcriptional activator protein A-LexA-V16 (PLV).

53. The method according to claim 48, wherein the first protein sequence comprises a C-terminal portion of ubiquitin (Cub) or a mutant thereof (CbM) and the second protein sequence comprises an N-terminal portion of ubiquitin (Nub) or a mutant thereof (NbM).

54. The method according to claim 48, wherein the DNA-sequence coding for the first membrane protein is selected from the group consisting of any bacterial membrane protein, any viral membrane protein, any oncogene-encoded membrane protein, any growth factor receptor or any eukaryotic membrane protein, or parts thereof.

55. The method according to claim 48, wherein the second membrane protein or the soluble protein, or part thereof, is encoded by a plasmid library.

56. The method according to claim 48, wherein the first membrane protein is a soluble protein attached artificially to the membrane.

57. A kit for detecting binding between a first membrane bound protein or part thereof and a second protein or part thereof which is either membrane bound or soluble comprising:
(a) a host cell containing at least one detectable gene (reporter gene) having a binding site for a transcriptional activator, such that the detectable gene

expresses a detectable product when the detectable gene is transcriptionally activated;
(b) a first vector (bait), which is maintained episomally, comprising a first site that can receive a first nucleic acid coding for a first membrane protein or part thereof such that when the first nucleic acid is inserted it becomes attached to the DNA sequence of a first module encoding *inter alia* a first protein sequence involved in intracellular protein degradation, the first module further comprising a nucleic acid for a transcriptional activator and a promoter;
(c) a second vector (prey), which is maintained episomally, comprising a second site that can receive a second nucleic acid coding for a second membrane protein or a soluble protein or part thereof such that when the second nucleic acid is inserted it becomes attached to the DNA sequence of a second module encoding *inter alia* a sequence protein sequence involved in intracellular protein degradation, wherein the second module further comprises a promoter; and optionally
(d) a plasmid library encoding second proteins or parts thereof,
wherein binding between said first membrane bound protein or part thereof and said second protein or part thereof is detected.

58. The kit according to claim 57, wherein the host cell is a yeast, a bacterium or a mammalian cell.

59. The kit according to claim 58, wherein the yeast is *Saccharomyces pombe* or *Saccharomyces cerevisiae*.

60. The kit according to claim 57, wherein the detectable gene can be activated by an activator comprising a short tagging module.

61. The kit according to claim 57, wherein the detectable gene can be activated by the artificial transcriptional activator protein A-LexA-V16 (PLV).

62. The kit according to claim 57, wherein the first protein sequence contains Cub or CbM and the second protein sequence contains Nub or NbM.

63. The kit according to claim 57, wherein the promoter in (b) is a CYC1 promoter or a CUP1 promoter.

64. The kit according to claim 57, wherein the DNA sequence coding for the first membrane protein is derived from any bacterial membrane protein, any viral membrane protein, any oncogene-encoded membrane protein, any growth factor receptor or any eukaryotic membrane protein, or parts thereof.

65. The kit according to claim 57, wherein the DNA sequence coding for the second protein is contained in a plasmid library.

66. A vector useful as a bait vector in the method of claim 48, wherein the vector comprises the following elements:
(a) a selection marker for propagation of the vector in E. coli;
(b) an origin of replication which allows propagation of the vector in E. coli;
(c) a further selection marker for propagation of the vector in yeast;
(d) an origin of replication which allows episomal propagation of the vector in yeast; and
(e) an expression cassette comprising the following elements:
(i) a promoter element;
(ii) a nucleic acid sequence encoding a leader selected from a signal sequence derived from a yeast integral membrane protein and a signal sequence, which confers fatty acid modification;
(iii) a nucleic acid sequence encoding Cub or CbM;
(iv) a nucleic acid sequence encoding a DNA binding protein; and
(v) a nucleic acid sequence encoding a transcriptional activator.

67. A host cell containing the vector of claim 66.

68. A method of identifying compounds, which method comprises using the kit of claim 57 to screen compounds for their ability to interfere with protein-protein interaction.

69. A method for providing a compound that can interfere with protein/protein

interaction, which method comprises:

(a) providing a host cell according to claim 67, the bait and prey polypeptides being selected such that they interact when expressed;

(b) incubating the host cell in the presence and absence of the compound(s) to be tested;

(c) measuring the difference in reporter gene expression between the incubation containing the compound(s) to be tested and the incubation free of the compound(s) to be tested; and optionally

(d) purifying or synthesizing the compound that can interfere with protein-protein interaction.

70. The method of claim 68, wherein said compound is a pharmaceutical drug.

71. The method of claim 48, wherein the bait vector is a low copy vector.

72. The method of claim 71, wherein the bait vector is present in 1 to 2 copies per cell.

73. The vector of claim 66, wherein said origin of replication in (d) is a CEN/ARS origin of replication.

74. The vector of claim 73, wherein the signal sequence encoded in (e)(ii) is: N-MGCTLSAEDKPGGP-C (SEQ ID No. 1).

75. The vector of claim 48, wherein said promoter in (b) is a promoter that confers low level expression.

76. The vector of claim 75, wherein said promoter in (b) is a CYC1 promoter or a CUP1 promoter.

9. Evidence Appendix

- **Stagljar et al.: PNAS 95:5187-92 (1998):** This evidence was entered in the record per applicants' Information Discloure Statement filed on September 1, 2005 and was considered by the Office on December 12, 2006.

- **Ehrhard et al.: Nature Biotech. 18: 1075-1079 (2000):** This evidence was entered in the record PTO-892, appended to the Office Action of October 3, 2007.

- **Fig. 1 of present application:** This illustration was entered with the filing of the present application on March 23, 2003, the U.S. national stage of which was entered on December 22, 2004.

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Genetics

A genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins *in vivo*

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ABSTRACT **A detection system for interactions between membrane proteins *in vivo* is described. The system is based on split-ubiquitin [Johnsson, N. & Varshavsky, A. (1994) *Proc. Natl. Acad. Sci. USA* 91, 10340–10344]. Interaction between two membrane proteins is detected by proteolytic cleavage of a protein fusion. The cleavage releases a transcription factor, which activates reporter genes in the nucleus. As a result, interaction between membrane proteins can be analyzed by the means of a colorimetric assay. We use membrane proteins of the endoplasmic reticulum as a model system. Wbp1p and Ost1p are both subunits of the oligosaccharyl transferase membrane protein complex. The Alg5 protein also localizes to the membrane of the endoplasmic reticulum, but does not interact with the oligosaccharyltransferase. Specific interactions are detected between Wbp1p and Ost1p, but not between Wbp1p and Alg5p. The new system might be useful as a genetic and biochemical tool for the analysis of interactions between membrane proteins *in vivo*.**

The analysis of interactions between proteins *in vivo* is essential for understanding their functions in the cellular context. The yeast two-hybrid system is a powerful method for the *in vivo* analysis of protein-protein interactions (1), but is limited to the analysis of soluble proteins or soluble domains of membrane proteins, i.e., interactions between integral membrane proteins cannot be studied. The split-ubiquitin system, which has recently been introduced for the analysis of interactions between soluble proteins, provides an alternative for the *in vivo* analysis of protein interactions (2). Ubiquitin is a conserved protein of 76 amino acids, which is usually attached to the N terminus of proteins as a signal for their degradation (3). The ubiquitin moiety is recognized by ubiquitin-specific protease(s) (UBP), resulting in the cleavage of the attached protein. The cleavage can be visualized with a stable reporter protein attached to the C terminus of ubiquitin (Fig. 1*A*). The ubiquitin-fusion can be divided and expressed in two parts, a N-terminal part (Nub*I*, amino acids 1–34, with *I* being isoleucine at position 13) and a C-terminal part of ubiquitin (Cub) (amino acids 35–76 of ubiquitin) fused to a reporter protein (2). Nub*I* and Cub-reporter assemble in the cell and form split-ubiquitin ([Nub*I*:Cub]-reporter). The split-ubiquitin is recognized by UBP, resulting in the cleavage of the reporter protein attached to Cub (Fig. 1*B*). Wild-type Nub*I* has a high affinity for Cub and assembles spontaneously to form a split-ubiquitin heterodimer. Replacement of Ile-13 of wild-type Nub*I* by alanine (Nub*A*) or glycine (Nub*G*) decreases the affinity between Nub and Cub (Fig. 1*C*). The association of Nub*G* with Cub-reporter is now dependent on additional protein contacts (Fig. 1*D*). The protein contacts can be provided by two test proteins, protein1 fused to Nub and protein2 to Cub-reporter. The detection of the cleaved reporter protein indicates interactions between protein1 and protein2.

The split-ubiquitin system was able to detect specific homotypic interactions between the zipper region of Gcn4p *in vivo* (2). The interaction between the two zippers was measured by immunoprecipitation and Western blot analysis of the cleaved reporter. We reasoned that the split-ubiquitin system would also be applicable to membrane proteins, provided that Nub and Cub are attached to parts of the protein, which localize to the cytosol. This is a prerequisite, because the necessary UBP is present in the cytosol and not in the lumen of the endoplasmic reticulum (ER) (4). Wbp1p is an essential component of the yeast oligosaccharyltransferase complex (5, 6) and in close proximity to Ost1p, another essential protein of the same complex (7–10). Both Ost1p and Wbp1p are type I transmembrane proteins with cytoplasmic C termini (9, 11). Alg5p is a type II transmembrane protein with both N and C termini in the cytoplasm, which synthesizes dolichol-phosphoglucose from dolicholphosphate and UDP-glucose (12). Alg5p is not known to interact with the oligosaccharyltransferase complex, but localizes to the membrane of the ER and is therefore suited as a control for the system. Using the oligosaccharyltransferase complex as a model, we established a detection system for interactions between integral membrane proteins. A transcription factor, protein A-LexA-VP16 (PLV) was used as the reporter molecule. PLV is liberated upon cleavage from Cub, hence able to activate *LacZ* and *HIS3* reporter genes, therefore providing a potentially useful tool for the screening of interaction between membrane proteins.

MATERIALS AND METHODS

Strains, Media. All constructs were expressed in the *Saccharomyces cerevisiae* strain L40 (*MATa trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ*) (13). pRS305(Δwbp1-Cub-PLV) was integrated at the single *Spe*I site into the *WBP1* gene resulting in strain YG0673 (*MATa trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ wbp1::pRS305(Δwbp1-Cub-PLV)*). YG0673

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This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: Nub, amino acids 1–37 of ubiquitin, either Nub*I* or Nub*A* or Nub*G*; Nub*I*, wild-type Nub, isoleucine at position 13; Nub*A*, Ile-13 replaced by alanine; Nub*G*, Nub Ile-13 replaced by glycine; Cub, C-terminal part of ubiquitin (amino acids 35–76). UBP, ubiquitin-specific protease(s); PLV, protein A-LexA-VP16 fusion; β-gal, β-galactosidase; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; ER, endoplasmic reticulum; ARS, autonomous replicating sequence.

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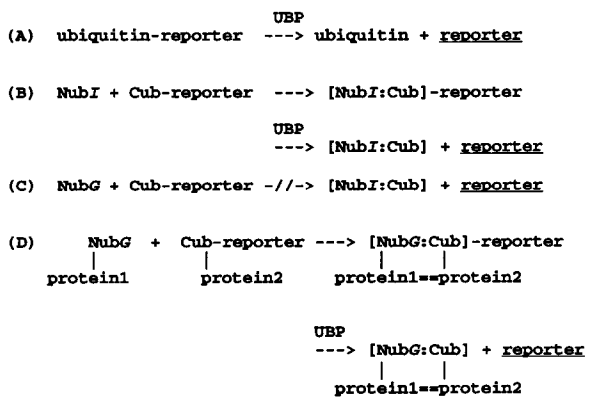

FIG. 1. Principle of the split-ubiquitin system. Split-ubiquitin is drawn as [Nub*I*:Cub] or [Nub*G*:Cub]. The liberated reporter protein is underlined. ":" indicates ubiquitin interaction; "=" indicates interaction between protein1 and protein2; for more information see the text.

was the recipient for different Nub-constructs. The *Escherichia coli* strain DH5α (*F⁻, endA1 hsdR17 (rk-mk⁺) supE44 thi recA1 gyrA96 relA1 f80d lacZΔM15*) (14) was used as the bacterial host for all plasmid constructions. Standard yeast media and techniques (15) were used. For selection of plasmids, dropout media containing all except the specified amino acids were used. All plasmids were transformed into yeast by lithium acetate transformation (16).

Construction of Plasmids. Sequences of the plasmid constructs and the detailed construction schemes are available upon request. All plasmids were verified by sequencing. The expression of all constructs was checked by Western blot analysis using suitable antibodies.

pRS305(Δwbp1-Cub-PLV). The *Apa*I-*Hin*dIII insert within pRS305 (17) encodes amino acids 251–430 of Wbp1p (Δ*wbp1*) (accession number P33767), fused to LEGSTMSG and the amino acids 35–76 of the yeast Ubi4p (Cub) (P04838), which in turn is linked by the sequence MHRSACGRMAG to the amino acids 151–275 of *Staphylococcus aureus* protein A (X96612) (18). The sequence ASGR links the protein A sequence to the amino acids 1–202 of LexA (J01643), which in turn is fused to EFPGIW and the amino acids 402–479 of VP16 (P04486). The sources of the DNAs were as follows: *WBP1*, p45–11 (11); Cub, pRS306(Δswp-Cub-dha) (N.J., unpublished data); protein A, p28NZZtrc (19); LexA-VP16, pLexA202+VP16 (gift from Alcide Barberis, University of Zürich).

pRS314(Nub*I*-ALG5). The region encompassing the *CUP1* promoter and Nub*G*GSTM was amplified from construct X (2) by PCR and fused to the ORF of the *ALG5* gene. The fusion protein consists of amino acids 1–37 of Ubi4p (Nub), followed by GGST and the 334 amino acids of Alg5p (P40350) (12). The fusion gene was inserted between the *Not*I-*Pst*I of pRS314. pRS314(Nub*A*-ALG5) and pRS314(Nub*G*-ALG5) were constructed by exchanging the small *Bam*HI fragment carrying *CUP1* and the mutated Nub-sequence from construct XII and construct XIII (2). The 2-μm plasmids pNub*I*-ALG5, pNub*A*-ALG5, and pNub*G*-ALG5 were constructed by exchanging the small *Pst*I-*Nru*I fragment of pAS2 (20) with the *Pst*I-*Sac*II fragment of the corresponding pRS314(Nub*I*-ALG5), pRS314(Nub*A*-ALG5), or pRS314(Nub*G*-ALG5).

pRS304(Δost1-Nub). A truncated *ost1* gene (codons 102–476) (P41534) (9) was fused to Nub*I*, Nub*A*, and Nub*G* in the vector pRS306 resulting in pRS306(Δost1-Nub*I*), pRS306(Δost1-Nub*A*), and pRS306(Δost1-Nub*G*). The encoded sequence after the C terminus of Ost1p is LEGGST followed by the amino acids 1–37 of Ubi4p (Nub), which in turn is fused to Thr-Leu-Glu and a stop codon. The Δost1-Nub*I*, Δost1-Nub*A*, and Δost1-Nub*G* gene fragments were transferred to the pRS304 vector to give pRS304 (Δost1-Nub*I*), pRS304(Δost1-Nub*A*), and pRS304 (Δost1-Nub*G*). For site-directed integration into the *OST1* locus, plasmids were linearized at the single *Sph*I site.

pOST1-Nub. The 5′-end of the *OST1* gene, including its promoter, was fused to Δost1-Nub*I*, Δost1-Nub*A*, and Δost1-Nub*G*, using the single *Sph*I site. The complete *OST1*-Nub*I*, *OST1*-Nub*A*, *OST1*-Nub*G* genes were used to replace the small *Eco*RI/*Sma*I fragment of the 2-μm plasmid pAS2 (20), resulting in the 2-μm plasmids pOST1-Nub*I*, pOST1-Nub*A*, and pOST1-Nub*G*.

β-Galactosidase (β-Gal) Activity Tests. Cells were grown for two days at 30°C on sterile Whatman filters on dropout agar plates lacking tryptophan and leucine and supplemented with 0.2 mM $CuSO_4$. The cells were permeabilized by dipping the filters into liquid nitrogen for 1 min. After thawing, the filters were overlaid with 1.5% agarose in 0.1 M $NaPO_4$-buffer (pH 7.0) containing 0.4 mg/ml 5-bromo-4-chloro-β-D-galactopyranoside (X-Gal), and incubated at 30°C for 1–23 h. For the quantitative assay, cells were grown in liquid dropout medium lacking tryptophan and leucine, supplemented with 0.2 mM $CuSO_4$ to an OD_{546} of 0.5–0.7. Cells (1.5 ml) were pelleted, washed once in Z buffer (113 mM Na_2HPO_4/40 mM NaH_2PO_4/10 mM KCl/1 mM $MgCl_2$, pH 7.0), suspended in 300 μl Z buffer. One hundred microliters were taken, lysed by 3 freeze/thaw cycles. Z buffer (700 μl) containing 0.27% (vol/vol) 2-mercaptoethanol and 160 μl 2-nitrophenyl-β-D-galactopyranoside (4 mg/ml in Z buffer) were added and incubated for 1–20 h at 30°C. Four hundred microliters of 0.1 M $NaCO_3$ was added, the sample centrifuged and the OD_{420} measured. β-Gal units were calculated as $1{,}000 \times OD_{420}/(OD_{546} \times \text{min})$.

Protein Extraction and Western Blot Analysis. Cells were grown at 30°C to OD_{546} of 0.3–1.2 in liquid dropout medium lacking tryptophan and leucine supplemented with 0.2 mM $CuSO_4$. Proteins were extracted essentially according to Horvath and Riezman (21). Cells were pelleted and suspended in 50 μl 1.85 M NaOH per 3 OD units of cells and incubated on ice for 10 min. The same volume of 50% trichloroacetic acid was added and proteins were precipitated by centrifugation for 5 min. The pellet was suspended in 50 μl of SDS/sample buffer containing 8 M urea. The proteins were dissolved after addition of 20 μl of 1 M Tris·base for 1.5 h at 37°C. Samples were centrifuged for 2 min and 10 μl extract was used for SDS/PAGE/Western blot analysis. Membranes were probed with peroxidase-IgG at 1:5,000 dilution. Protein A-fusion proteins were detected by enhanced chemiluminescence (Amersham). The amount of protein loaded was verified by Coomassie blue staining of the SDS gels.

RESULTS

Experimental Design. Wbp1p is a transmembrane protein of the ER and a subunit of the oligosaccharyltransferase complex (5). To analyze interactions between Wbp1p and other proteins, we elongated the C terminus of Wbp1p by Cub as the interaction module, followed by a modified transcription factor (PLV). The final fusion protein was termed Wbp1-Cub-PLV (Fig. 2*A*). The rationale was to anchor the transcription factor to the membrane of the ER, thereby preventing it from activating genes in the nucleus (Fig. 2*B*). Coexpression of Wbp1-Cub-PLV protein with a noninteracting Nub-fusion-protein, like Nub*G*-Alg5p, does not lead to activation (Fig. 2*C*). Coexpression of an interacting protein fused to Nub*G* results in the assembly and the recognition of the split-ubiquitin heterodimer by UBP(s). The protease liberates PLV, which probably enters the nucleus by diffusion and activates then *lacZ* and *HIS3* reporter genes (Fig. 2*D*).

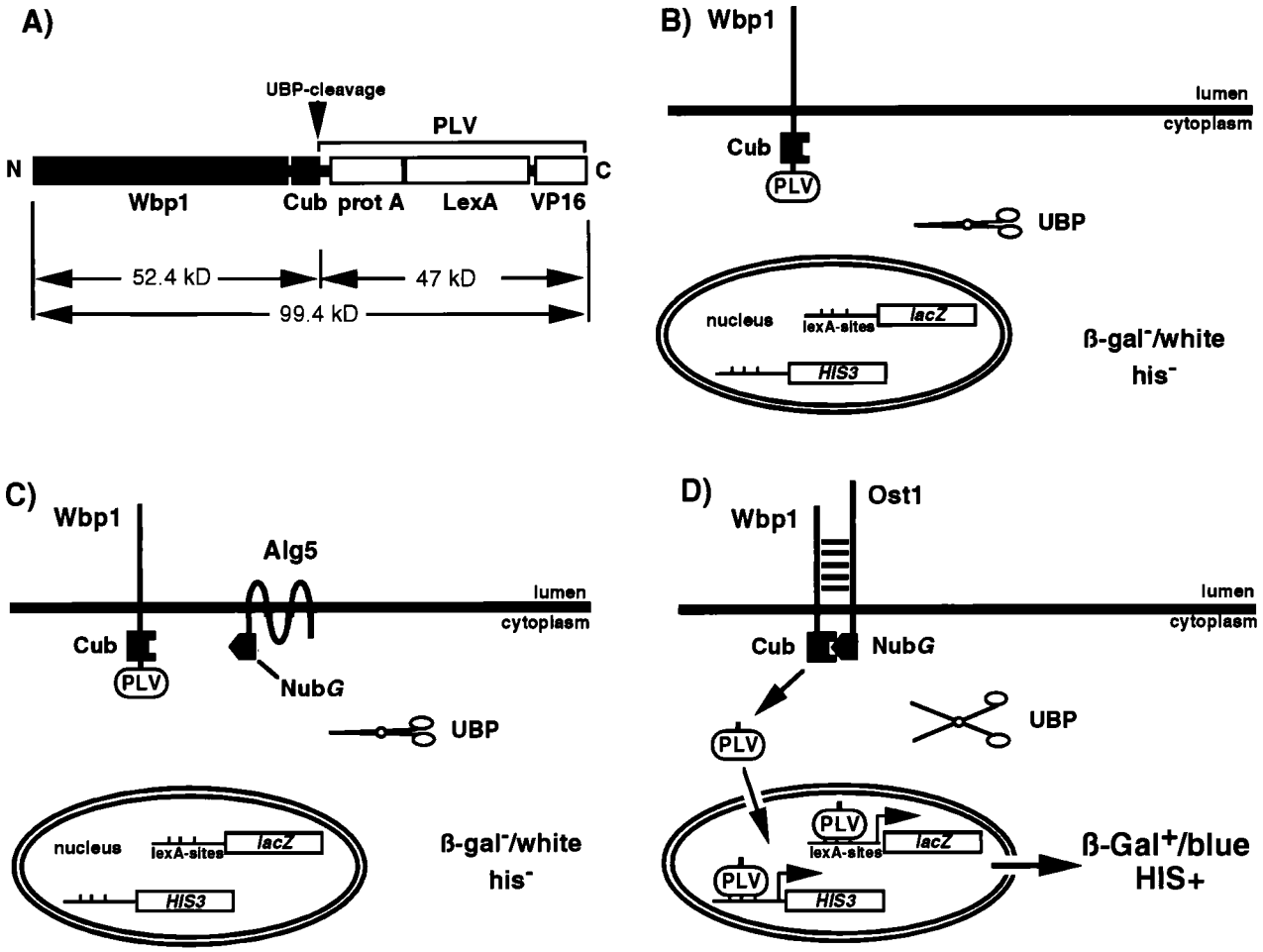

FIG. 2. Design of Wbp1-Cub-PLV fusion protein and the principle of detection of the interaction. (*A*) The structure of the mature Wbp1-Cub-PLV fusion protein. Cleavage by the UBP(s) occurs at the C terminus of Cub, cleaving the Wbp1-Cub-PLV fusion protein of ≈100 kDa into Wbp1-Cub (52 kDa) and PLV (47 kDa). (*B*) Expression of PLV as a fusion to the ER membrane protein Wbp1p prevents the transcription factor from gene activation in the nucleus. Cleavage of Wbp1-Cub-PLV by UBP does not occur (solid scissors) in the absence of Nub, the cells are white in the presence of X-Gal and are His auxotrophs. (*C*) Coexpression of Wbp1-Cub-PLV with the noninteracting Nub*G*-Alg5p does not lead to formation of the split-ubiquitin heterodimer, nor cleavage by UBP (solid scissors) and gene activation. (*D*) Interaction between Wbp1 and Ost1 results in formation of the split-ubiquitin heterodimer. The heterodimer is recognized and cleaved by the UBP (open scissors), liberating PLV. PLV can enter the nucleus by diffusion and bind to the LexA-binding sites leading to activation of transcription of the *lacZ* and *HIS3* reporter genes. This results in blue cells in the presence of X-Gal and growth of the cells on agar plates lacking histidine.

The protein A sequence from *Staphylococcus aureus* contains two IgG-binding domains, which allow easy and sensitive detection of the fusion protein as well as of the cleaved product. The LexA-VP16 cassette consists of the entire DNA-binding protein LexA followed by the transcriptional activation domain of VP16 (22). LexA-VP16 can activate reporter genes with LexA binding sites in the promoter region. The *WBP1-Cub-PLV* fusion gene was generated by site-directed integration of a PLV cassette containing a 5′-truncated Δ*wbp1* gene (Δ*wbp1-Cub-PLV*) into the genomic *WBP1* locus. Thus, only the modified Wbp1-Cub-PLV, but no wild-type Wbp1p, was present in the cell. The insertion of Cub-PLV at the C terminus of Wbp1p did not inactivate the essential Wbp1p function in the oligosaccharyltransferase complex (11). Nub*I*, Nub*A*, and Nub*G* were fused to the 3′-end of the ORF of a 5′-truncated Δ*ost1* gene. The resulting fusion genes were integrated into the *OST1* locus to give only one active Ost1 copy, expressing Ost1-Nub*I*, Ost1-Nub*A*, or Ost1-Nub*G*. The generated cells were viable. Hence, the addition of Nub to the C terminus did not inactivate the essential Ost1 protein (9). To analyze the competition between the wild-type Ost1p and Ost1-Nub, we placed the complete fusion genes OST1-Nub*I*, OST1-Nub*A*, or OST1-Nub*G* on a 2-μm plasmid and expressed each of them together with the wild-type chromosomal *OST1* gene. As a control, Nub*I*, Nub*A*, or Nub*G* were fused to the 5′-end of the ORF of the *ALG5* gene. The fusion of Nub to the N terminus of Alg5p did not inactivate the protein. All constructions using 2-μm vectors resulted in 10- to 20-fold overexpression of the respective Nub-fusion protein (data not shown).

Interaction of Wbp1-Cub-PLV with Ost1-Nub and Nub-Alg5p. Ost1p is a member of the oligosaccharyltransferase complex and expected to interact with Wbp1p (7–10). In contrast, Alg5p, dolicholphosphoglucose synthetase (12), is not expected to interact with Wbp1p. To test for interactions, Ost1-Nub or Nub-Alg5 were coexpressed with Wbp1-Cub-PLV in a *S. cerevisiae* strain carrying *lacZ* and *HIS3* reporter genes under the control of LexA-binding sites (13). Cells were tested for β-gal activity after permeabilization on Whatman filters with X-Gal.

Expression of Wbp1-Cub-PLV with Nub*I*-Alg5 or Nub*A*-Alg5 resulted in blue cells in the presence of X-Gal. In contrast, Nub*G*-Alg5 showed very little β-gal activity (Fig. 3, lanes 1 and 2). It has been shown with soluble proteins that Nub*I*-fusions associate with Cub independent of additional protein-protein contacts, whereas interactions of Cub with Nub*A* or Nub*G* are dependent on additional contacts (4). The absence of β-gal activity in the case of Nub*G*-Alg5 suggests that there are no specific interactions between Wbp1p and Alg5p.

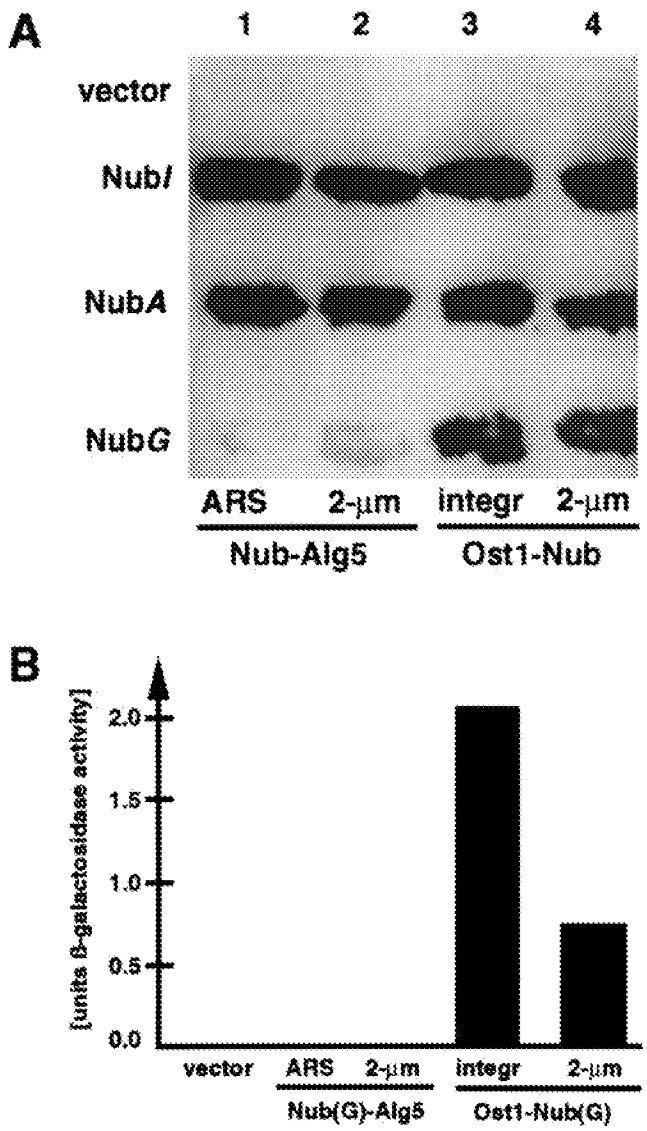

FIG. 3. β-Gal activity of cells expressing Wbp1-Cub-PLV together with Nub-fusion proteins. (*A*) YG0673 cells expressing Wbp1-Cub-PLV and (*i*) Nub*I*-Alg5, Nub*A*-Alg5, or Nub*G*-Alg5 from a CEN/ARS plasmid; (*ii*) Nub*I*-Alg5, Nub*A*-Alg5, or Nub*G*-Alg5 from a 2-μm plasmid; (*iii*) Ost1-Nub*I*, Ost1-Nub*A*, or Ost1-Nub*G* from an integrated fusion gene (no wild-type Ost1p present in the cell); (*iv*) Ost1-Nub*I*, Ost1-Nub*A*, or Ost1-Nub*G* from a 2-μm plasmid in presence of the wild-type Ost1p. As negative control, YG0673 was transformed with the vector pRS314. Cells were grown on Whatman filters, permeabilized, and incubated in the presence of X-Gal. Expression of β-gal resulted in blue cells. (*B*) Quantitative β-gal assay of YG0673 cells expressing Wbp1-Cub-PLV together with the vector, low copy number pRS314(Nub*G*-ALG5) (ARS), Nub*G*-ALG5 (2 μm), OST1-Nub*G* (integrated fusion gene, no wild-type OST1 present), and OST1-Nub*G* (2 μm, wild-type OST1 present). Shown are the results of one out of three independent experiments.

Expression of Ost1-Nub fusions together with Wbp-Cub-PLV resulted in strongly blue cells with Ost1-Nub*I*, Ost1-Nub*A*, and, in contrast to Nub*G*-Alg5, also with Ost1-Nub*G* (Fig. 3*A*, lanes 3 and 4). We were interested to see, whether the modified proteins were able to compete with the wild-type protein for interactions. In the case of the integrated Ost1-Nub fusion, cells have to utilize the modified Ost1-Nub, because the Ost1 protein is essential. The situation is different, if Ost1-Nub is expressed in the presence of the wild-type Ost1p. Preliminary experiments had indicated that a high level of expression of Ost1-Nub is needed for successful competition with the unmodified Ost1p (data not shown). Therefore, high-copy number 2-μm yeast plasmids were used for the expression of Ost1-Nub in presence of the wild-type Ost1p (Fig. 3, lane 4). The blue color indicates that the modified Ost1-Nub proteins were able to compete with the wild-type Ost1p. As a control, Nub-Alg5 was expressed by the same vector system, resulting in blue color in the case of Nub*I*- and Nub*A*-, but not Nub*G*-Alg5 (Fig. 3, lane 2). As a further control, expression of Wbp-Cub-PLV with the empty vector plasmid did not give any β-gal activity. In summary, the β-gal activity in the case of Ost1-Nub*G* demonstrated the expected specific interactions between Wbp1 and Ost1. The absence of β-gal activity in the case of Nub*G*-Alg5 served as a stringent control for the system. This absence of activity is unlikely due to a general inaccessibility of Nub*G*-Alg5 to Wbp1-Cub-PLV, because expression of Nub*I*-Alg5 and Nub*A*-Alg5 results in β-gal activity.

We confirmed the results by the use of a quantitative assay with 2-nitrophenyl-β-D-galactopyranoside as a substrate for β-gal (Fig. 3*B*). Ost1-Nub as the sole Ost1 protein in the case of the integrated fusion gene in the cell led to high β-gal activity. This was reduced to about one-third if Ost1-NubG was expressed from a 2-μm plasmid in the presence of the wild-type Ost1p. No β-gal activity was detectable after 1 h of incubation with the vector or Nub*G*-Alg5p expressed from low copy number [autonomous replicating sequence (ARS)] or high copy number plasmid (2 μm). We also checked the growth behavior of cells expressing Wbp1-Cub-PLV together with Ost1-Nub or Alg5-Nub on agar plates lacking histidine (Fig. 4). Expression of Wbp1-Cub-PLV with the empty vector resulted in His− cells. Coexpression of Wbp1-Cub-PLV with Ost1-Nub*I*, -Nub*A*, and -Nub*G* results in His+ cells. Growth of cells expressing Nub*G*-Alg5p on agar plates lacking histidine was reduced compared with Ost1-Nub*G*.

Interaction-Mediated Cleavage of Wbp1-Cub-PLV *in Vivo*. We were interested to see whether the β-gal activity and the HIS+ phenotype correlated with the actual cleavage of the Wbp1-Cub-PLV reporter protein. Therefore, protein extracts were prepared from cells expressing Wbp1-Cub-PLV with either Ost1-Nub or Nub-Alg5 and determined by Western blot analysis and probing with peroxidase-IgG (Fig. 5). The Wbp1-Cub-PLV fusion protein was easily detected at a relative molecular mass of 95 kDa (lane 1), which is in good agreement with the calculated molecular mass of 99.4 kDa. The cleaved PLV reporter was visible upon coexpression with Nub-constructs as a band migrating at ≈50 kDa (calculated 47 kDa) (Fig. 5, lanes 2–6 and 8–13). The band migrating at a position

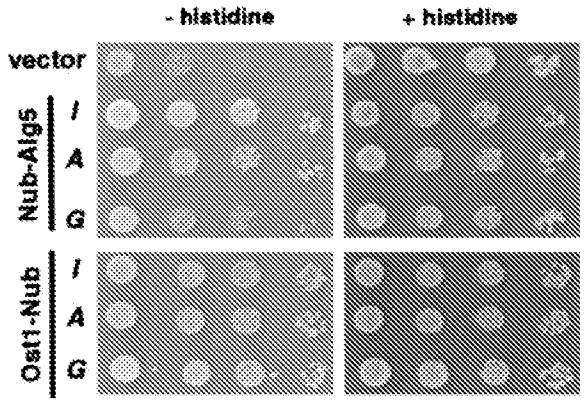

FIG. 4. Growth of cells expressing Wbp1-Cub-PLV with various Nub-fusions on agar plates lacking histidine. YG0673 cells expressing Wbp1-Cub-PLV and either Nub*I*-Alg5, Nub*A*-Alg5, or Nub*G*-Alg5 from a 2-μm plasmid or expressing Ost1-Nub*I*, Ost1-Nub*A*, or Ost1-Nub*G* from a 2-μm plasmid were grown to logarithmic phase, spotted in serial 10-fold dilutions on selective agar plates containing 0.2 mM $CuSO_4$ +/− histidine and were incubated for 5 days at 30°C. YG0673 transformed with the vector pRS314 served as negative control.

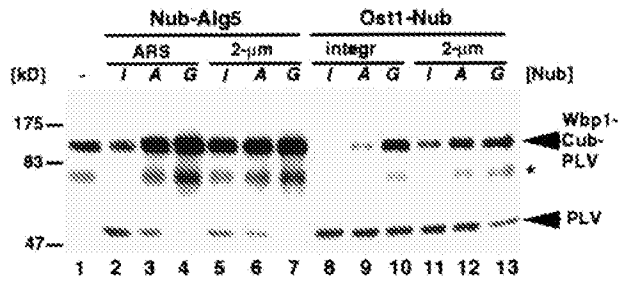

FIG. 5. Western blot analysis of cells expressing Wbp1-Cub-PLV together with Nub-fusions. YG0673 cells expressing Wbp1-Cub-PLV with the vector (lane 1); with either Nub*I*-Alg5, Nub*A*-Alg5, or Nub*G*-Alg5 from a CEN/ARS plasmid (lanes 2–4); or from a 2-μm plasmid (lanes 5–7). YG0673 expressing Ost1-Nub*I*, Ost1-Nub*A*, or Ost1-Nub*G* as the sole source of Ost1 in the cell (lanes 8–10, integr) or from a 2-μm plasmid in presence of wild-type Ost1p (lanes 11–13). Cells were grown to logarithmic phase in selective medium; proteins were extracted and determined by Western blot analysis and probing with peroxidase-IgG as described in *Materials and Methods*. *, Unspecific degradation product.

of ≈75 kDa may arise by an unspecific degradation of the fusion protein. Cleavage of Wbp1-Cub-PLV upon coexpression of Nub-Alg5 constructs occurred in the case of Nub*I*-Alg5 (Fig. 5, lanes 2 and 5), which was reduced with Nub*A*-Alg5 (Fig. 5, lanes 3 and 6) and almost completely abolished with Nub*G*-Alg5 (Fig. 5, lanes 4 and 7). These findings are in good correlation with the strong reduction of β-gal activity in the case of Nub*G*-Alg5 (Fig. 3). Integration of OST1-Nub*I* into the OST1 gene resulted in complete cleavage of the Wbp1-Cub-PLV (lane 8). Cleavage was reduced, but still occurring using Ost1-Nub*A* and Ost1-Nub*G* (lanes 9 and 10). In agreement with the β-gal activity observed in Fig. 3, expression of Ost-Nub in the presence of the wild-type Ost1p also produced significant amounts free PLV from Wbp1-Cub-PLV (lanes 11–13). Importantly, free PLV is also found with Ost1-Nub*G* (lane 13). Therefore, although the Ost1-Nub fusion proteins had to compete with the endogenous wild-type Ost1p, they were still able to interact with Wbp1-Cub-PLV protein (compare lanes 8–10 with 11–13). The use of the dhfr-ha (dihydrofolate reductase) reporter cassette described in ref. 2 as a reporter results in the same cleavage pattern as with the Wbp1-Cub-PLV in Fig. 5 (data not shown).

DISCUSSION

We were interested in generating a genetic system allowing the analysis of membrane protein interactions *in vivo*. The term "interaction" is hereby used for describing either physical contact between two proteins or close spatial arrangement, for example the presence of two proteins in the same protein complex. The split-ubiquitin system has been used to detect specific interactions between known soluble proteins (2). Some of the advantages of the split-ubiquitin system are: (*i*) *in vivo* and *in situ* detection of protein–protein interaction, there is no need for nuclear localization; (*ii*) small modules (Nub and Cub) attached to linker sequences are used to detect interactions thereby minimizing potential steric hindrance; (*iii*) the possible detection of transient interactions; and (*iv*) the detection of interactions is mediated via cleavage by UBP and not by transcription. Therefore, proteins could be tested, which carry by themselves transcriptional activating or repressing sequences. However, in contrast to the two-hybrid technique, no system suitable for the screening of interactions was available for the split-ubiquitin.

Using well characterized membrane proteins Wbp1p and Ost1p of the *S. cerevisiae* oligosaccharyltransferase, we demonstrate that the split-ubiquitin also works with membrane proteins. In addition, the introduction of the PLV-cassette as the reporter molecule provides a potentially useful tool for the screening of interactions. PLV is not known to contain a nuclear localization signal and does not lead to gene activation, when fused to the ER membrane protein Wbp1p (Fig. 3 *A* and *B*). Although strict evidence is lacking, it is unlikely that the PLV transcription factor is simply inactivated by the fusion to Wbp1p. First, the transcriptionally active module LexA-VP16 is already extended by the protein A sequence. Second, overexpression of WBP1-Cub-PLV or the expression of a soluble Cub-PLV results in gene activation in the absence of Nub (data not shown). Therefore, we believe that the inactivation of PLV is because of its compartmentalization to the ER membrane, as shown in Fig. 2. As a consequence, the Cub-PLV system might also work for testing for interactions of soluble proteins, provided they are fused to membrane anchors.

It has been shown that Nub*A*-fusions to soluble proteins can assemble with Cub only in the presence of additional protein-protein contracts (4). In our experiments, Nub*A*-Alg5 assembles unspecifically with Wbp1-Cub-PLV. This is understandable in the light that the membrane protein interactions are limited to two dimensions of the lipid bilayer, thereby increasing the probability of unspecific reactions. However, Nub*G*-Alg5 cannot assemble with Wbp1-Cub-PLV.

The modified split-ubiquitin system could be used to screen a library of cDNAs or yeast genomic DNA linked to Nub*G* as 5′ or 3′ fusions for interaction with a protein of interest. Two other systems have been published recently, which potentially might allow also the testing of interactions between membrane proteins, but which have not been examined with membrane proteins so far. One system uses activation of plasma membrane anchored yeast Ras bound to GDP by human SOS protein (23), and the second system uses fusions to β-gal for α-complementation of β-gal (24). To our knowledge, our study represents the first report of a system capable of the *in situ* analysis of interactions between membrane proteins.

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Use of G-protein fusions to monitor integral membrane protein–protein interactions in yeast

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The control of protein-protein interactions is a fundamental aspect of cell regulation. Here we describe a new approach to detect the interaction of two proteins in vivo. By this method, one binding partner is an integral membrane protein whereas the other is soluble but fused to a G-protein γ-subunit. If the binding partners interact, G-protein signaling is disrupted. We demonstrate interaction between known binding partners, syntaxin 1a with neuronal Sec1 (nSec1), and the fibroblast-derived growth factor receptor 3 (FGFR3) with SNT-1. In addition, we describe a genetic screen to identify nSec1 mutants that are expressed normally, but are no longer able to bind to syntaxin 1a. This provides a convenient method to study interactions of integral membrane proteins, a class of molecules that has been difficult to study by existing biochemical or genetic methods.

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All biological processes require precise control of protein activity. Proteins can be regulated by post-translational modifications, altered localization, or association with regulatory subunits or other components of a supramolecular structure (e.g., ribosome, cytoskeleton).

In the past, protein–protein interactions have typically been studied using biochemical techniques such as cross-linking, co-immunoprecipitation, and co-fractionation by chromatography. A disadvantage of these techniques is that interacting proteins often exist in low abundance and are therefore difficult to detect. Moreover, once an interaction is detected, the newly identified protein still must be isolated and sequenced, before the gene can be identified. Another disadvantage is that these methods do not immediately provide information about which domains of a protein are involved in the interaction.

To address technical difficulties associated with the biochemical characterization of protein–protein interactions, alternative genetic methods have been developed. One such method is the yeast two-hybrid system, wherein two proteins are fused to either the DNA-binding domain or the transcription-activation domain of Gal4 (refs 1,2). If the two proteins interact, the function of Gal4 is reconstituted (Fig. 1). Transcriptional activation can be detected using the appropriate promoter and a reporter gene, such as *lacZ* (encodes β-galactosidase). This approach allows the rapid detection of protein binding partners, including the relevant interacting domains, and immediately provides the gene that encodes the identified interacting proteins. Various permutations of the two-hybrid method have been described, including the split-ubiquitin system[3,4], the SOS-recruitment system[5,6], dihydrofolate reductase complementation[7,8], and β-galactosidase complementation[9,10].

Although the yeast two-hybrid system has greatly facilitated the study of protein–protein interactions, there are some situations where it is not suitable. For instance, the method relies on the interaction of two proteins in the nucleus of the cell, so the method is not useful for the study of most integral membrane proteins. Moreover, if one of the proteins is a transcriptional activator, it may itself induce transcription of the reporter gene. Finally, the yeast two-hybrid system requires that both proteins be expressed as fusion proteins, resulting in the possible loss of function.

Here we describe a method to monitor protein–protein interaction, using the well-characterized G-protein signaling pathway as the readout. G-protein-coupled receptors can respond to hormones, neurotransmitters, odors, and light. Receptor activation triggers a conformational change in the G-protein α-subunit, exchange of GDP for GTP, and dissociation of Gα from the G-protein βγ-subunits. Depending on the system, either Gα or Gβγ can activate downstream effectors, until GTP is hydrolyzed and the protein reverts to the inactive conformation (Fig. 1). In yeast, pheromone stimulation leads to activation of a G protein composed of the products of the *GPA1* (Gα), *STE4* (Gβ), and *STE18* (Gγ) genes. Gβγ in turn activates a kinase signaling cascade that culminates in growth arrest, new gene transcription, cell fusion, and mating[11].

In the method described here, two protein-binding partners have been tested: syntaxin 1a with nSec1, and FGFR3 with SNT-1. Syntaxin 1a was chosen because it has a well-characterized function in synaptic vesicle fusion, and it is a proven drug target. nSec1 binding prevents syntaxin 1a assembly with the "SNARE core complex," which is needed for bilayer fusion. An inhibitor of syntaxin is used therapeutically to treat muscle spasms and spasticity that result from inappropriate neurotransmitter release[12,13]. FGFR3 was selected because it plays a key role in cell differentiation and proliferation, and mutations of the receptor are associated with birth defects and cancer[14,15]. Binding of fibroblast growth factor to FGFR3 leads to receptor dimerization, tyrosine phosphorylation (including receptor autophosphorylation), and the recruitment of cytoplasmic signaling proteins that transmit the signal. Other cytoplasmic proteins, such as SNT-1, bind permanently to the intracellular domain of the receptor[16,17].

The ability to monitor the activity of these proteins expressed in their native form (not as a hybrid) and localized at the cell membrane (not in the nucleus) should prove useful in screening for mutants, drugs, or other proteins that alter their binding properties.

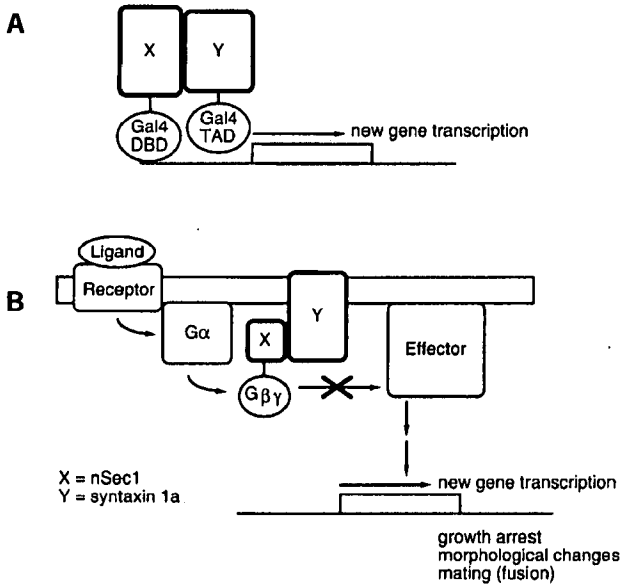

Figure 1. Use of G-protein fusions to monitor integral membrane protein–protein interactions in yeast. (A) In the traditional two-hybrid method the interaction between protein X and protein Y occurs in the nucleus. Reconstitution of the Gal4 DNA-binding domain (DBD) and transcription-activation domain (TAD) leads to induction of a reporter gene. (B) In the G-protein fusion method, the interaction between protein X and protein Y occurs at the membrane, and sequesters Gβγ. Disruption of G-protein signaling leads to reduced transcription of a reporter gene and failure to undergo growth arrest. Drugs or mutations that disrupt binding of X and Y will restore G-protein signaling.

Results

Expression of syntaxin 1a and nSec1-Gγ. Our central goal was to be able to monitor the binding of integral membrane proteins to their cytoplasmic protein targets in vivo. Our approach was to convert these interactions into a G-protein-mediated event. With fusion of a cytoplasmic binding partner to the G-protein γ-subunit, high-affinity interactions should disrupt G-protein-dependent changes in gene transcription and cell growth (Fig. 1).

As a first test of this approach, we expressed syntaxin 1a and a fusion of nSec1 with Gγ in yeast. We initially examined whether expression of syntaxin 1a or the nSec1-Gγ fusion alone would interfere with G-protein signaling, using the pheromone-dependent growth inhibition (halo) assay. A *ste18*Δ mutant (Gγ-deficient) strain was transformed with plasmids expressing nSec1-Gγ, syntaxin 1a, or Gγ (Ste18) alone. Cells were plated and then exposed to different amounts of synthetic α-factor spotted onto filter disks. After 48 h, cells expressing nSec1-Gγ exhibited a clear zone of growth inhibition, comparable to that seen with Gγ (Fig. 2A). These results indicate that attachment of a heterologous protein to the N terminus of Gγ does not alter Gβγ assembly or signaling in vivo. In contrast, co-expression of syntaxin 1a and nSec1-Gγ yielded considerably more turbid halos, indicating a potent inhibition of Gβγ signaling (Fig. 2A). Presumably nSec1-Gγ is capable of forming a functional dimer with Gβ, but one that binds preferentially to syntaxin 1a and is therefore unable to activate a signaling pathway leading to growth arrest.

Table 1. nSec1 mutations that disrupt binding to syntaxin 1a

S42F	Contact site
M51K, K294R	Contact site, contact region
D112N	Contact region
I482T, K524M	Hinge region

To confirm that binding is specific, we tested another isoform of syntaxin (syntaxin 4) that does not recognize nSec1[18]. In this case, co-expression with nSec1-Gγ yielded normal halos, comparable to Gγ alone (data not shown). The expression and membrane association of nSec1-Gγ or syntaxin 1a was not altered under any of the conditions tested, as shown by immunoblotting (Fig. 2B).

To corroborate the results of the halo assay, and to provide a more quantitative assessment of the change in pheromone signaling, we performed a reporter transcription assay (Fig. 2C). For these experiments we used the *lacZ* reporter gene under the control of the pheromone-inducible promoter from *FUS1* (refs 19,20). As shown in Figure 2C, expression of nSec1-Gγ yielded β-galactosidase activities even higher than that seen with wild-type Gγ. In contrast, co-expression of syntaxin 1a with nSec1-Gγ resulted in a marked decrease in the maximum level of induction, with no change in EC_{50} for pheromone induction. These data are consistent with the halo assay above, indicating that the nSec1-Gγ can function in place of Gγ, but preferentially binds to syntaxin 1a.

Expression of FGFR3 and SNT-Gγ fusion. To determine if the G-protein fusion method can be used to monitor the interaction of other proteins, we tested a second pair consisting of FGFR3 and SNT-1. SNT-1 was selected because binding is independent of receptor activity[21]. FGFR3 was selected because it does not appear to be toxic to the host yeast cell, unlike several other tyrosine kinases (see below)[22,23]. Finally, FGFR3 is an attractive drug target, since the FGF signaling pathway is permanently activated in some cancer cells[24,25].

As shown in Figure 3A, expression of the SNT-Gγ fusion yielded normal halos, comparable in size to Gγ alone. However, cells co-expressing FGFR3 and SNT-Gγ exhibited more turbid halos, indicating a loss of G-protein signaling. These results were corroborated by the transcription reporter assay (Fig. 3B). Again, expression of Gγ or SNT-Gγ yielded nearly equivalent β-galactosidase responses, whereas co-expression of FGFR3 and SNT-Gγ yielded a marked decrease in the maximum level of induction. These results mirror those described above, using nSec1 and syntaxin 1a.

Genetic screen for nSec1 mutants that block binding to syntaxin 1a. A particular advantage of any yeast-based assay is the ability to carry out simple genetic screens on a large scale. Thus the approach described above could also be used to screen for mutations that modulate the interaction between any two protein-binding partners, even those not normally present in yeast. As an example, mutations in nSec1 that disrupt binding to syntaxin 1a could easily be isolated, by screening for the reacquisition of pheromone responsiveness. A unique feature of this approach is that such mutants must not interfere with expression or overall folding of the protein, since the Gγ moiety must retain the ability to bind Gβ, even if nSec1 activity is lost.

To isolate nSec1 mutants that no longer bind syntaxin 1a, the nSec1-Gγ fusion was amplified by polymerase chain reaction (PCR) using conditions designed to increase misincorporation of nucleotides ("error prone PCR"). The amplified products were then co-transformed with the original plasmid, which had been digested so as to remove the entire nSec1 open reading frame. After transformation into yeast, recombination of the DNA fragments allows plasmid replication and cell growth on selective media. Resulting colonies were replica stamped to plates either with or without high concentrations of α-factor. Rare α-factor-sensitive colonies were then restreaked, and retested for pheromone sensitivity using the halo assay (58 colonies out of 15,000 screened). Finally, to confirm that the activity was conferred by the plasmid, episomal DNA was prepared from 13 colonies, amplified in *Escherichia coli*, and used to retransform the original *ste18*Δ strain. After these manipulations, four candidate mutants were selected for sequencing (Table 1).

Sequencing of the nSec1-Gγ mutants revealed single-site substitutions at position 42 (serine to phenylalanine, $nSec1^{S42F}$) and 112 (aspartic acid to asparagine, $nSec1^{D112N}$). The remaining mutants con-

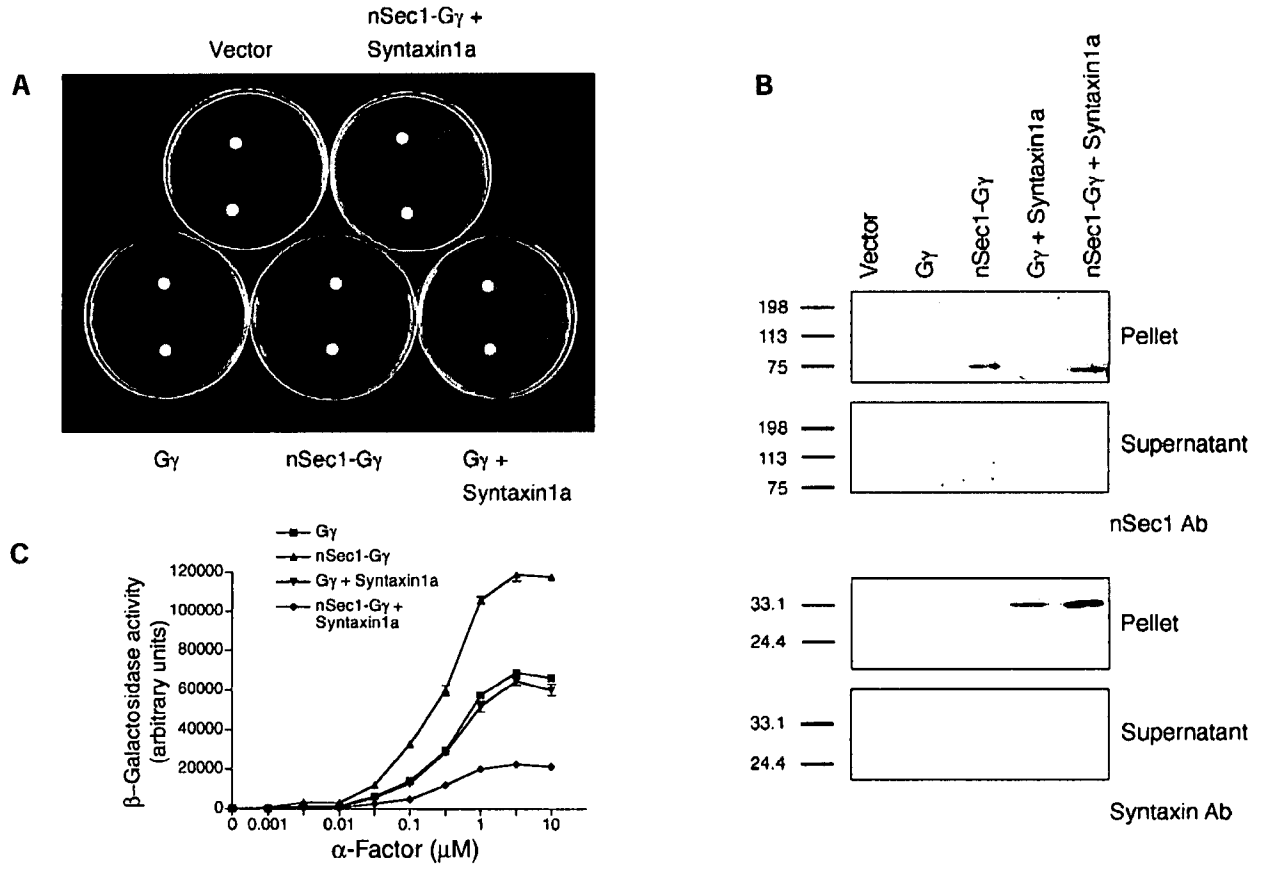

Figure 2. Detection of syntaxin 1a binding to nSec1. (A) Halo assay. Gγ-deficient cells (*ste18Δ* mutant) were transformed with vectors containing no insert ("vector"), Gγ, nSec1-Gγ fusion, syntaxin 1a, or syntaxin 4 (not shown) as a negative control. Cells were plated and exposed to filter disks containing 20 or 48 μg α-factor pheromone for 48 h, and then photographed. (B) Immunoblot. To confirm expression of nSec1 and syntaxin, cells were lysed, centrifuged to resolve membrane ("pellet") and cytosolic ("supernatant") fractions, and resolved by gel electrophoresis. Immunoblots were probed with antibodies to nSec1 (top panels) or syntaxin (bottom panels). Mobility of molecular weight standards is indicated. (C) Reporter transcription assay. Cells were treated with the indicated concentrations of α-factor, and β-galactosidase activity was determined using a pheromone-responsive *FUS1* promoter-lacZ reporter construct. Data shown are typical of two to five independent experiments performed in triplicate. Error bars, ±s.e.

tained two substitutions, at 51 (methionine to lysine) and 294 (lysine to arginine) (nSec1$^{M51K, K294R}$) or at 482 (isoleucine to threonine) and 524 (lysine to methionine) (nSec1$^{I482T, K524M}$). All of these mutations can be rationalized in the context of the recently described crystal structure of the nSec1–syntaxin 1a complex[13]. This analysis revealed that nSec1 is composed of three domains, arranged in an arch that surrounds a portion of syntaxin 1a. Residues in the first and third domains form direct contacts with syntaxin 1a, and these contacts are largely polar or complementary in charge. Three of the mutants isolated in our screen alter a contact-site amino acid (S42F, M51K) of the first domain or an amino acid within a contact region of the first (D112N) or third (K294R) domain. The remaining mutant affects residues distal to the contact interface, but that are part of a hinge region needed to form the arch that surrounds syntaxin 1a.

Discussion

All cell processes involve highly regulated protein–protein interactions. One particularly well-characterized example involves receptor–G protein coupling, and the consequent dissociation and reassociation of G-protein subunits. Here, we describe an adaptation of this signaling apparatus that permits the detection of other protein–protein interactions in vivo. Our method is conceptually similar to the yeast two-hybrid method, where two binding partners are fused to the DNA-binding and transcriptional-activation domains of Gal4, respectively (Fig. 1A). In our method, one of the proteins is fused to the G-protein γ-subunit. Interaction between the two test proteins disrupts G protein-dependent signaling, affecting such easily measured events as gene transcription and cell growth (Fig. 1B). In principle, other effects of G-protein activation could also be monitored, such as phosphorylation, protein translocation, cell morphogenesis, and fusion (mating). The availability of multiple signaling assays will likely reduce the incidence of false positives. In contrast, other well-known signaling pathways, such as that controlled by the small G-protein Ras, have not been nearly as well delineated in yeast[5,6].

A major advantage of our method is that it can be used to detect protein–protein interactions at the plasma membrane. This is significant because about 40% of all proteins (including many important drug receptors) are thought to be anchored in the lipid bilayer, and are unlikely to enter the nucleus[26]. Thus, our approach could be used to identify drugs that bind receptors at the cell surface, and regulate the activity of some target protein inside the cell, through changes in receptor conformation. Some examples include receptor tyrosine kinases, ion channels, transporters, virus receptors, antigen receptors, and cell adhesion molecules.

A second advantage of our approach is that only one of the two binding partners needs to be expressed as a fusion protein. A protein in its native form is more likely to exhibit normal folding and ligand

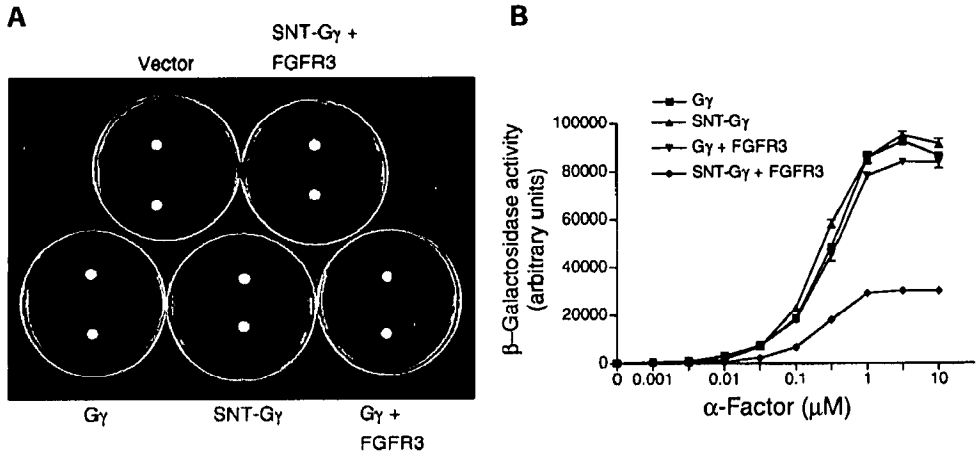

Figure 3. Detection of FGFR3 binding to SNT-1. (A) Halo assay. Gγ-deficient cells (*ste18Δ* mutant) were transformed with vectors containing no insert ("vector"), Gγ, SNT-Gγ fusion, or FGFR3, as indicated. Cells were plated and exposed to filter disks containing 20 or 48 μg α-factor pheromone for 48 h, and then photographed. (B) Cells were treated with the indicated concentrations of α-factor, and β-galactosidase activity was determined using a pheromone-responsive *FUS1* promoter-lacZ reporter construct. Data shown are typical of two to five independent experiments performed in triplicate. Error bars, ±s.e.

binding properties. Even some native proteins cannot be functionally expressed in yeast, however. For instance, we have attempted to express the FGFR2 and HER-2/*neu* receptor without success. One alternative may be to express these proteins in cultured mammalian cells instead of in yeast.

Another goal for the future is to identify new binding partners for integral membrane proteins, including syntaxin 1a and the FGFR3. The construction of cDNA libraries in standard two hybrid vectors has been extremely useful in this regard. A similar approach could be used to screen cDNAs fused to Gγ, or to screen for competitive inhibitors of known binding partners fused to Gγ.

Finally, we are interested in identifying new drugs that regulate FGFR3 or syntaxin 1a activity. The ability to find mutations that disrupt protein–protein interactions suggests that drugs with similar properties might be identified[27]. Moreover, a screen for mutants can usually be adapted into a screen for drugs, using the same readout. Syntaxin 1a is a known substrate for *Clostridium botulinum* toxin, and this agent is used therapeutically to treat conditions of overactive muscle contraction, such as spasticity in children with cerebral palsy, or spasms in patients with multiple sclerosis, stroke, or spinal cord injury[12]. Thus, the identification of new inhibitors of syntaxin 1a could lead to new drugs for the treatment of human disease.

In summary, we describe an approach to detect interaction between two proteins in vivo. We have demonstrated the utility of the method for two different protein-binding partners, and for carrying out genetic screens for mutants that are expressed normally but are no longer able to recognize their binding partner. This approach could be used to monitor the interactions between any two proteins in a cell, under various physiological and pharmacological conditions.

Experimental protocol

Strains, media, and plasmid construction. Standard methods for the growth, maintenance, and transformation of yeast and bacteria, and for the manipulation of DNA, were used throughout[28]. The yeast *Saccharomyces cerevisiae* strain used in this study was MHY6 (MATa *ura3-52 lys2-801*^{am} *ade2-101*^{oc} *trp1-63 his3- 200 leu2-1 ste18::LEU2*) (provided by Jeremy Thorner, University of California Berkeley).

STE18 was PCR amplified using a 5′ oligonucleotide containing an *Eco*RI site followed by sequence encoding MAHHHHHHASM (original start codon). The PCR product was ligated into the yeast expression vector pRS314-GAL (ref. 29) (amp^r, *CEN/ARS, TRP1, GAL1/10* promoter) to yield pRS314-GAL-H6-STE18. To prepare Gγ fusions, the coding sequence of rat nSec1 or human SNT-1 (with a C-terminal triple *myc* epitope tag, provided by Mitchell Goldfarb, Mt. Sinai School of Medicine) was PCR amplified and ligated into pRS314-GAL-H6-STE18. Full-length rat syntax 1a was PCR amplified and ligated into pRS316-ADH (amp^r, *CEN/ARS, URA3, ADH1* promoter and termination sequence)[30]. Mouse FGFR3, either wild type (data not shown) or containing the TDII-type mutation[31], was PCR amplified, and then ligated by co-transformation and homologous recombination into vector pRS423-GAL (amp^r, 2μ, *HIS3, GAL1/10* promoter, *CYC1* terminator) or pRS426-GAL (amp^r, 2μ, *URA3, GAL1/10* promoter, *CYC1* terminator).

Mutagenesis of the GAL-nSec1-H6-STE18 cassette was carried out using "error-prone PCR," as described[32]. The amplified products were recombined with pRS314-GAL-nSec1-H6-STE18, which had been digested with *Eco*RI so as to remove the entire nSec1 open reading frame, by co-transformation and nutritional selection. Resulting colonies were replica stamped to plates either with or without ≥40 μM of α-factor. Rare α-factor-sensitive colonies were then restreaked, and retested for pheromone sensitivity using the halo assay. Plasmid DNA was prepared from each colony, amplified in *E. coli*, and used to retransform the original *ste18Δ* strain, and retested by the halo assay.

Cell disruption, membrane fractionation, and immunoblot analysis. For preparation of whole-cell lysates, cell pellets were resuspended in 1× sample buffer for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), boiled for 10 min, and subjected to glass bead vortex homogenization for 2 min. Fractionated cell lysates were prepared as described[30]. Protein extracts were resolved by 8% or 12% SDS–PAGE and transferred to nitrocellulose. Blots were probed with antibodies against nSec1 (supplied by Pietro De Camilli, Yale University) or syntaxin (supplied by Colin Barnstable, Yale University). Blots were also performed using antibodies against syntaxin 4 (Chemicon International, Temicula, CA), FGFR3 (C-15; Santa Cruz Biotechnology Inc., Santa Cruz, CA), or FRS2 (H-91; Santa Cruz Biotechnology Inc.) (data not shown). SNT-1 is homologous to mouse FRS2. Antibody detection was achieved using horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad Laboratories, Hercules, CA) or goat anti-rabbit IgG (Bio-Rad Laboratories) and colorimetric[28] or chemiluminescence detection (New England Nuclear, Boston, MA; Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions.

Pheromone response assays. The pheromone-dependent growth inhibition assay (halo assay) was performed as described[30].

For pheromone-dependent reporter-transcription assays[19], mid-log phase cells were aliquoted (90 μl) to a 96-well plate, and mixed with 10 μl of α-factor for 90 min, in triplicate. β-galactosidase activity was measured by adding 20 μl of a freshly prepared solution of 83 μM fluorescein di-β-D-galactopyranoside (10 mM stock in dimethyl sulfoxide; Molecular Probes, Eugene, OR), 137.5 mM PIPES pH 7.2, 2.5% Triton X-100, and incubating for 90 min at 37°C. The reaction was stopped by the addition of 20 μl 1 M Na_2CO_3, and the resulting fluorescence activity was measured at 485 nm excitation, 530 nm emission. Because of differences in instrument calibration, sample number, and the like, data from each experiment are presented as arbitrary units rather than absolute values.

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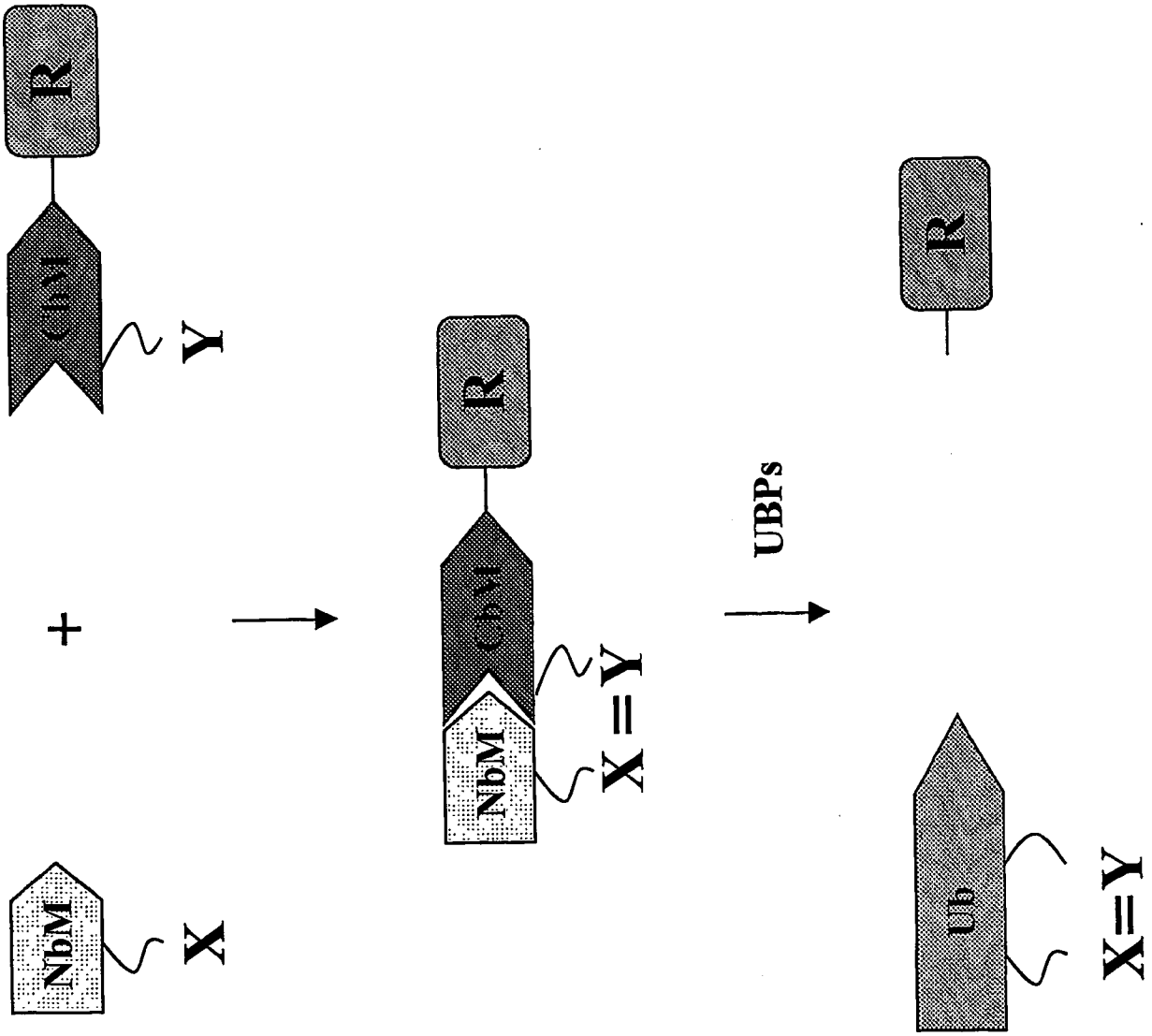
Figure 1



10. Related Proceedings Appendix

None